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A comparative study between the values of lecithin, sphingomyelin, lysolecithin, the L/S index, and the CLEMENTS test in amniotic fluid

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It has been known for only a few years that the fetal lung is capable of synthetizing phospholipid substances with surfactant properties. Since AVERY and MEAD's work [1], which was later verified by other authors, a correlation is known to exist between the newborn respiratory distress syndrome (R.D.S.) due to hyalin membranes and the deficit of surfactant substances.

In 1971, Gluck et al. [15] found a statistically significant correlation between the presence of a low lecithin/sphingomyelin concentration ratio (L/S index), and the appearance of neonatal respiratory distress. They observed that an **L/S index below 1.5 is accompanied by respiratory distress syndrome (RDS) in nearly 90% of the cases, while with an index above 2 this syndrome is seldom present.** These results were later widely confirmed by other authors using the same method, but whose results differed from those of GLUCK; i.e., DONALD (63%) [10], GOLDSTEIN (36%) [16], SPELLACY (33%) [26] and HOBBINS (25%) [17]. However, the techniques employed to obtain these results require the availability of a very well equipped laboratory, which means that their use is limited to large centres. Likewise, the necessary time for the majority of the techniques described is at least 2–3 hours. For this reason, CLEMENTS et al. [8] in 1972 described the test wich bears his name, also known as the "**Shake Test**", wich is based on the capability of the amniotic fluid of maintaining bubbles after shaking with ethanol in an ionic medium (saline).

The advantage of this test is its great simplicity, low cost and quick results, which can be either positive, intermediate or negative. The negative results usually are accompanied by RDS; the percentage probability of this finding varies according to the respective author. In this investigation the percentage of simultanous occurence was 63% (CABERO et al., [6]) and according to previous investigations, when the results were

Curriculum vitae

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positive, the gestation was in at least the 35th week in all of the patients (CABERO et al. [2]).

BHAGWANANI, FAHMY and TURNBULL [7], have described a modification of the CLEMENTS Test, basing their experiment on the same principles.

Up to the present, numerous works have been published concerning the clinical importance of the L/S index and/or lecithin values in amniotic fluid in relation to the possible presence of hyaline membrane disease in the neonatal period [9, 11, 13, 15, 18]. The interest in the CLEMENTS Test and its clinical application has also been emphasized in the literature [8, 24]. Comparative studies between the values of lecithin or the L/S index and the CLEMENTS Test have also been published [5, 9, 13, 19, 20, 21, 22, 23, 27].

In this investigation a **comparative study** has been made **between this test and the absolute values of lecithin, lysolecithin, sphingomyelin, and the L/S ratio in amniotic fluid.**

1 Material and methods

Ninety-two samples of amniotic fluid were taken from 85 patients, with or without associated pathology, beginning from the 31st week some of whom were in labor. In 32 cases the amniotic fluid was obtained by amniocentesis according to FREDA's technique [14] or by means of para-umbilical puncture after having localized the placenta by ultrasound. In the other 62 cases, the sample was obtained by means of amniotomy through amnioscopy, or through the open-end catheter used to register the intra-amniotic pressure during labor.

The following procedure was used in applying the CLEMENTS Test: 3 cc of amniotic fluid were collected and processed within 15 minutes in the same obstetrical area and at room temperature.

Pipetted volumes of 1, 0.75, 0.50, 0.25 and 0.20 ml of amniotic fluid were added respectively to a battery of five test tubes, each measuring 8–12 × 100 mm, and numbered 1 to 5. A 0.9% saline solution was added to tubes 2, 3, 4 and 5 in the respective volumes of 0.25, 0.50, 0.75 and 0.80 ml. Finally, 1.0 ml of 95% ethanol was added to each of the five tubes.

The tubes were then closed and shaken simultaneously for 15 seconds to obtain a homogeneous agitation in all samples. Afterwards they were placed in a vertical position for 15 minutes before being read. This consisted of observing the presence of small bubbles in the meniscus of the air/liquid interphase, a positive value being given to the tubes in which the bubbles either did not encircle the ring completely, or were absent. There were 3 possible values: positive, intermediate, and negative. Positive results were those in which at least the first three tubes were positive. The negative result is when none of the tubes were positive and the intermediate result is when the first tube or the first and second tubes were positive.

All the amniotic fluid contaminated by meconium, blood, vaginal secretions, or antiseptics were discarded, since all of them cause false positive results.

1.1 Determination of phospholipids

For the determination of lecithin, sphingomyelin and lysolecithin, 5 ml. of amniotic fluid were withdrawn and immediately put into a closed bottle containing 30 ml of chloroform-methanol 2:1 and kept in a refrigerator until the moment of determination. It was observed that the phospholipids remained unchanged for at least one month when preserved in this manner.

The extraction was accomplished according to the classic method of ZÖLLNER using chloroform-methanol 2:1. After adding water it was left standing overnight in order to obtain a better phase separation. The chloroform extract was then dried by means of a rotary evaporator.

The residue, dissolved in chloroform, was applied to a silica gel slide (using an exactly measured quantity of liquid) with a SHANDOM applicator, which allows the formation of a 2.5 cm long band. The chromatogram was developed in a chamber saturated to a height of about 18 cm, using MÜLDNER's elution which gives an excellent separation of sphingomyelin and lysolecithin (elution: 30% chloroform-methanol-ammonia at 14:6:1).

When the development was finished, the slide was placed in an oven at 100°C for two minutes and the stains made visible by pulverizing in an iodine saturated solution in petroleum ether. The stains were marked and once the iodine had been eliminated, they were scraped carefully. The powder was put into 2 ml syringes containing a piece of cotton which had previously been extracted in the Soxhlet with chloroform to eliminate all lipids and then had been compressed by the piston into a compact mass 2–3 cm high. The silica gel lipid was eluted with 4 × 2.5 ml of chloroform/methanol/acetic acid/water (50:39:1:10) [3]. An aliquot was dried in a tube and digested by perchloric acid. The phosphate was determined in the usual way with ammonium molybdate.

2 Results

The results of the CLEMENTS Test were classified in three groups: positive, intermediate and negative. A correlation between each of these groups has

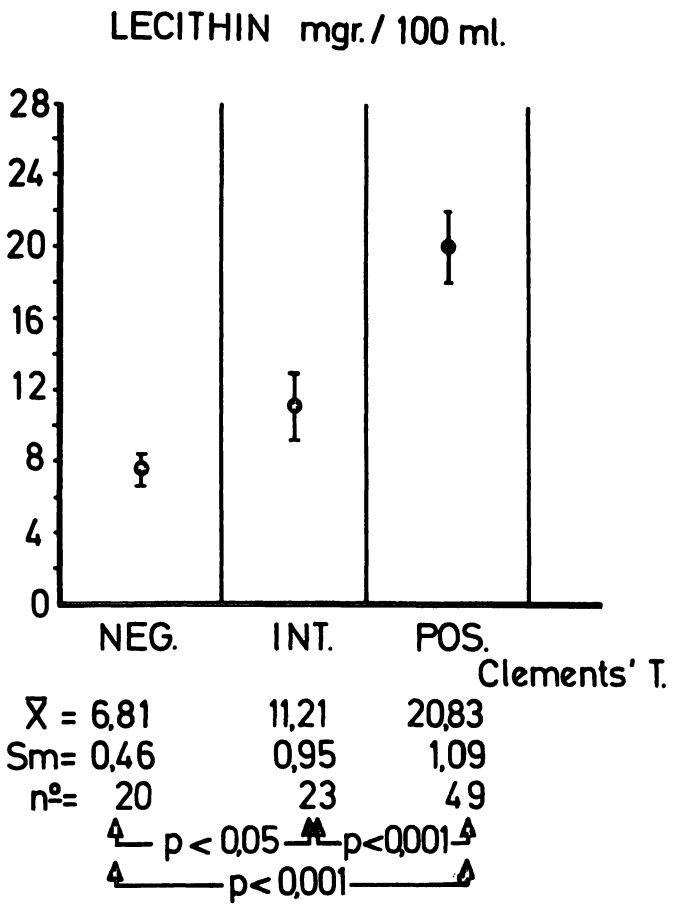

Fig. 1. Correlations between lecithin values and the results of the CLEMENTS Test. The high values of lecithin are due to the extraction method used (BHAGWANANI et al. [24]).

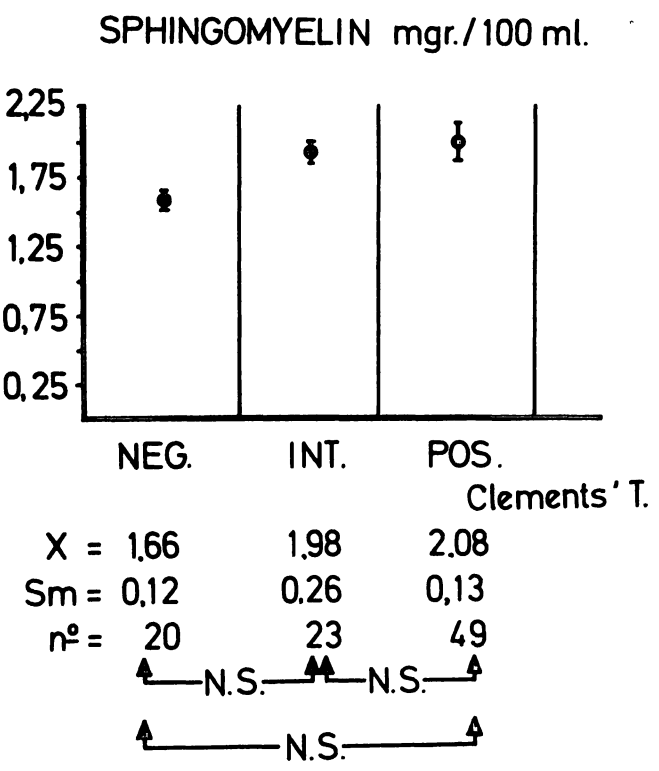

Fig. 2. Correlation between sphingomyelin and the results of the CLEMENTS Test.

been established for the values of lecithin sphingomyelin, lysolecithin and the L/S index. For this comparison the mean and the standard error of the mean were first found, followed by an analysis of variance arrived at by comparing the means of each of these groups. The statistical significance was obtained by means of the Student t-Test.

Fig. 1 shows the correlation between the values of **lecithin**, expressed in mg/100 ml, and the results of the CLEMENTS Test. The values obtained were 6.87 ± 0.46 for the negative group of the CLEMENTS Test, 11.21 ± 0.95 for the intermediate group, and 20.83 ± 1.09 for the positive group. The comparison between them shows **statistically significant differences** with a $p < 0.05$ between the negative and intermediate groups, a $p < 0.001$ between the positive and intermediate groups, and a $p < 0.001$ between the positive and negative groups.

Fig. 2 illustrates the relation between the values of **sphingomyelin** and the CLEMENTS Test. The results were 1.66 ± 0.12; 1.98 ± 0.26 and 2.08 ± 0.13 respectively, for the negative, intermediate and positive groups, without statistically significant differences between them.

Fig. 3 shows the relation between the values of **lysolecithin**, expressed in mg/100 ml. and the results of the CLEMENTS Test, in its three possibilities: negative intermediate and positive. As can be seen, the mean values of lysolecithin: 1.37 ± 0.12; 1.74 ± 0.25 and 2.02 ± 0.14 respectively were found for each group of the CLEMENTS Test. There were no statistically significant differences between the negative and intermediate, and the positive and intermediate groups. On the contrary, the difference was statistically significant between the positive and negative values, with a $p < 0.05$.

In Fig. 4 a correlation can be seen between the **L/S index** and the CLEMENTS Test. The values for the negative, intermediate and positive groups were: 4.42 ± 0.38; 6.44 ± 0.56 and 10.97 ± 0.58 respectively, with no statistically significant differ-

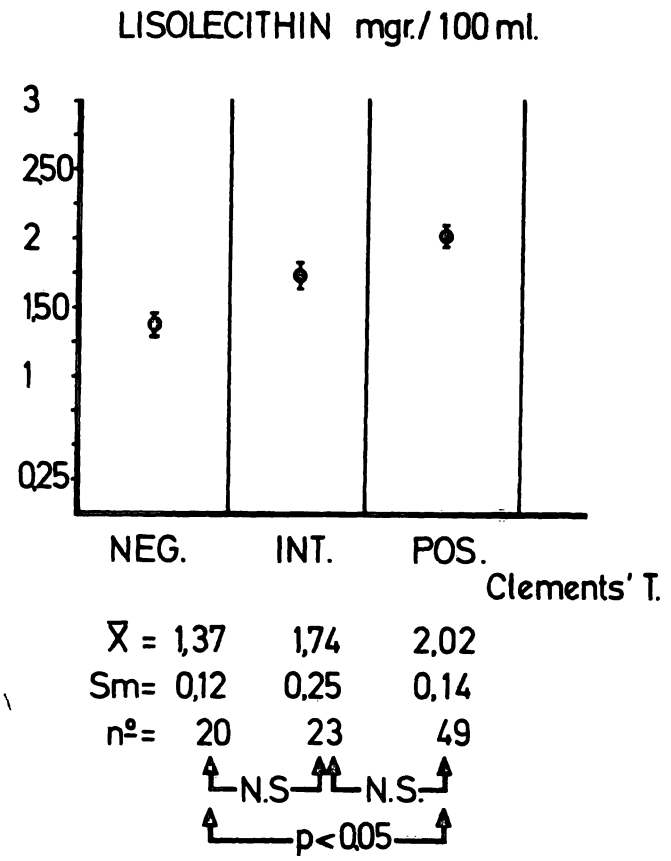

Fig. 3. Correlation between lysolecithin values and the results of the CLEMENTS Test.

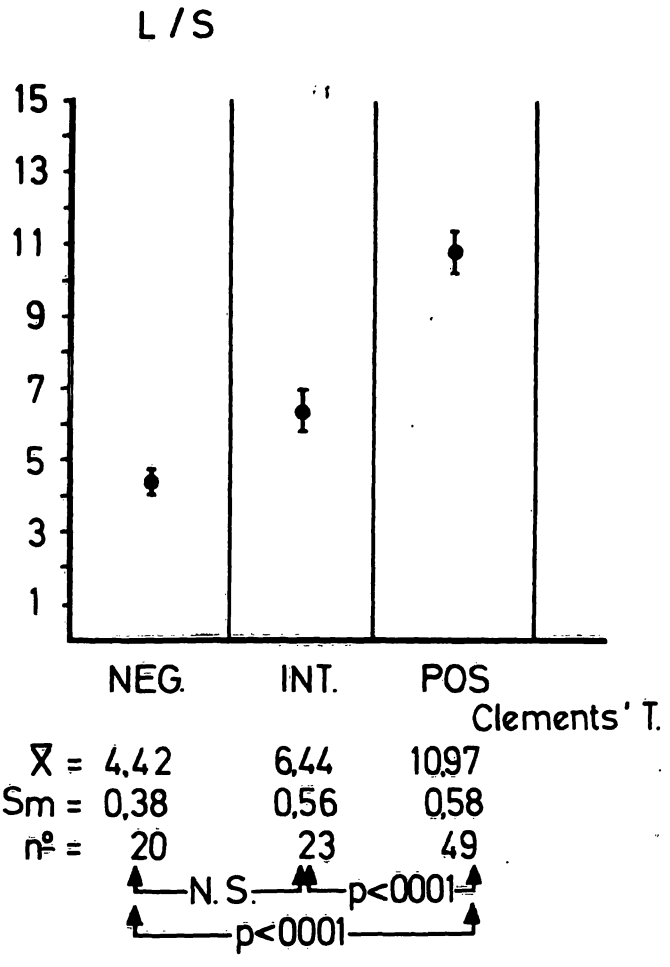

Fig. 4. Correlation between the L/S ratio values and the results of the CLEMENTS Test.

ences between the negative and intermediate groups. The differences were statistically significant between the intermediate and positive groups and negative and positive groups.

Comments

These results are in agreement with those of other authors [5, 19, 20, 21, 22, 23, 27] thereby permitting the assertion that **the values of the different phospholipids studied, with the exception of sphingomyelin, have a clear correlation with the results given by the CLEMENTS Test.**

At the same time it was ascertained that when the CLEMENTS Test was positive, it had a statistically significant relationship with high values of lecithin and lysolecithin, as well as with a high L/S index. There was no correlation with the sphingomyelin values.

These compounds form a part of the phospholipid complex of amniotic fluid. SCARPELLI [25] has already proposed that they come from the fetal lung, since these compounds were also observed in this organ at a concentration similar to that of amniotic fluid. It was only after the studies of BIGGS et al. [4] and ENHÖRNING et al. [12] that there was absolute evidence that these substances come from the fetal lung.

On the other hand, the lack of correlation between sphingomyelin and the CLEMENTS Test detracts from the evaluation of this substance.

A clear correlation exists between the CLEMENTS Test value and the concentration of lecithin in amniotic fluid. This emphasizes the fact that the two parameters have a common base, and that the first depends on the second. Definitely, the high values of phospholipids, which are capable of diminishing surface tension, are conducive to a stabilization of the bubbles and consequently to

a positive CLEMENTS Test. Therefore, according to these results the CLEMENTS Test has an important significance in obstetrics. It must be remembered that the rather sophisticated technique of determining phospholipids in amniotic fluid demands a considerable amount of equipment and time. The validity and practical simplicity of the CLEMENTS test suggests its acceptance as a routine test. Within the context of pregnancy monitoring, it is a useful and informative method for a given clinical situation, and can be used as a screening method.

Summary

The relationship between the concentration of several phospholipids in amniotic fluid and the ocurrence of neonatal RDS was established some years ago.
However the methods used for the determination of those substances are sophisticated and time consuming. They require specialized equipment and take at least 2 or 3 hours. CLEMENTS described a semiquantitative method which overcomes these disadvantages.
The present study compared the concentration of lecithin, shingomyelin, lysolecithin and the L/S ratio against the results obtained when processing the same samples with the CLEMENTS Test. The results are expressed in mg/100 ml. for the different phospholipids and the test was evaluated as positive, intermediate or negative.
A statistically significant correlation has been found between the values of lecithin, lysolecithin and L/S ratio and the results of the CLEMENTS Test, respectively. No correlation could be found with sphingomyelin (Figs. 1–4).
It is concluded that the CLEMENTS Test can be a useful tool as a screening test available to any obstetrical centre.

Keywords: Amniotic fluid, cephalin, CLEMENTS-Test, lecithin, L/S ratio, lysolecithin, neonatal respiratory distress syndrome, phospholipids, shake test, sphingomyelin.

Zusammenfassung

Eine Vergleichsstudie zwischen der Wertigkeit von Lecithin, Sphingomyelin, Lysolecithin, dem L/S-Quotienten und dem CLEMENTS-Test im Fruchtwasser

Vor einigen Jahren wurde der Zusammenhang zwischen der Konzentration verschiedener Phospholipide im Fruchtwasser und dem Auftreten eines RDS bei Neonaten entdeckt. Die Methoden für die Bestimmung dieser Substanzen sind jedoch aufwendig und zeitraubend. Sie erfordern Spezialausrüstungen und dauern mindestens 2–3 Stunden. CLEMENTS beschrieb eine semiquantitative Methode, die diese Nachteile nicht aufweist. In der vorliegenden Studie wird ein Vergleich angestellt zwischen der Konzentration von Lecithin, Sphingomyelin, Lysolecithin und der L/S-Ratio und jenen Ergebnissen, die mit dem CLEMENTS-Test in denselben Fruchtwasserproben erzielt wurden. Die Ergebnisse werden in mg/100 ml für die verschiedenen Phospholipide dargestellt und der CLEMENTS-Test wurde als positiv, intermediär oder negativ bewertet.
Es fand sich eine signifikante Korrelation zwischen den Lecithin-, Lysolecithin-Werten und der L/S-Ratio einerseits und den Ergebnissen des CLEMENTS-Testes andererseits. Für Sphingomyelin ließ sich keine Korrelation mit dem CLEMENTS-Test nachweisen (Figs. 1–4). Aus den Resultaten wird der Schluß gezogen, daß der CLEMENTS-Test, der in jedem geburtshilflichen Zentrum zur Verfügung steht, als Screening-Test von Nutzen sein kann.

Schlüsselwörter: Amnionflüssigkeit, Cephalin, CLEMENTS-Test, RDS, Lecithin, L/S-Quotient, Lysolecithin, Phospholipide, Schüttel-Test, Sphingomyelin.

Résumé

Etude comparative des valeurs de lécithine/sphingomyeline, lysolecithine et test de CLEMENTS dans le liquide amniotique

Les relations qui existent entre la concentration de plusieurs phospholipides du liquide amniotique et la survenue d'une détresse respiratoire néonatale ont été établies depuis plusieurs années.
Malheureusement les méthodes utilisées pour la détermination de ces substances sont longues et élaborées: elles nécessitent une technologie très spécialisée et durent au moins 2 à 3 heures.
CLEMENTS a décrit une méthode semi-quantitative qui évite ces inconvénients.
La présente étude compare les concentrations de lécithine, de Sphingomyéline, de Lysolécithine et du rapport L/S avec les résultats du test de CLEMENTS appliqué aux mêmes prélèvements.
Les résultats sont exprimés en mg pour 100 ml pour les différents phospholipides et le test de CLEMENTS a été

évalué comme positif, intermédiaire ou négatif. Une corrélation statistique/ment significative a été trouvée entre les valeurs de Lécithine, lécithine et rapport L/S d'une part, et d'autre part les résultats du test de CLEMENTS. Aucune corrélation n'a été trouvée avec la Sphingomyéline (Figs. 1–4).

La conclusion est que le test de CLEMENTS peut être un outil utile comme test de dépistage dans n'importe quel centre obstétrical.

Mots clés: Céphaline, Detresse respiratoire du nouveau-né, Lécithine, Liquide amniotique, Lysolécithine, Phospholipide, Rapport L/S, Test d'agitation, Test de Clements.

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